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A genome-wide scan and *HCRTR2* candidate gene analysis in a European cluster headache cohort

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Abstract—*Objective:* To investigate the molecular genetic basis of cluster headache (CH), using a genome-wide scan and candidate gene strategy. *Methods:* Northern European CH families and a case-control cohort of Danish, Swedish, and British origin (total n = 259 sporadic CH patients), including 267 control subjects matched for ancestry, participated in the study. A genome-wide genetic screen using approximately 400 microsatellite markers was performed for five informative Danish CH families. Additional markers were typed for those loci generating statistical evidence suggestive of linkage, together with genotypes for 111 individuals from further Danish and Italian kindreds. Sporadic CH patients and controls were investigated by association analysis for variation in the candidate gene, *HCRTR2*. Finally, complete *HCRTR2* sequencing was undertaken for eight independent probands. *Results:* Potential linkage was identified at four possible disease loci in Danish kindreds, yet no single chromosome location generated a lod or NPL score of recognized significance. No deleterious sequence variants of the *HCRTR2* gene were detected by comparison to wild-type sequence. Association of the *HCRTR2* gene was not replicated in this large dataset, even when the data were stratified into distinct populations. *Conclusions:* Cluster headache is a complex genetic disorder, with possible phenotypic and genetic heterogeneity compounding attempts at gene identification.

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Cluster headache (CH) is characterized by unilateral, periodic attacks of excruciating pain, accompanied by autonomic dysfunction.[1,2] In <10% of patients, chronic CH features persist.[3] The disease has an estimated prevalence of 1/500,[4-6] and displays marked sex bias (female:male ratio <1:2.5 to 3.5).[1,7,8] CH is considered to be a neurovascular disorder with activation of hypothalamic, trigeminovascular, and cranial parasympathetic systems.[9,10] Altered neurohormone levels[11-14] indicate central activation, and PET studies reveal ipsilateral inferior hypothalamic gray matter changes during attacks.[10,15] Pain relief by deep brain stimulation of the posterior hypothalamus further implicates central activation processes in disease pathogenesis.[16,17]

Several observations support a genetic predisposition to CH. Relative risk for disease is increased,[8] while pedigree analysis indicates autosomal dominant segregation, with reduced penetrance.[18] Incomplete concordance for monozygotic twins with CH[8,19] suggests a complex etiology with environmental involvement.

The molecular basis of CH remains poorly characterized. Mitochondrial mutations seen in two subjects[20,21] may be chance findings.[22,23] Case-control analyses of the calcium channel gene *CACNA1A* and the nitric oxide synthase genes *NOS1*, *NOS2A*, and *NOS3*, while good biologic candidates, revealed no allelic association.[24-28] Furthermore, linkage to the *CACNA1A* locus has been excluded in a CH family.[29]

The neuropeptides hypocretin-1 and -2, expressed within the posterolateral hypothalamus,[30] activate the hypocretin receptor 2 (HCRTR2) and regulate the sleep-wake cycle, with hypocretin system deficiencies associated with narcolepsy.[31] A recent study in Italian sporadic patients identified a single *HCRTR2* polymorphism associated with CH.[32] Here, we present results from a genome-wide scan in CH families and seek replication of association to the *HCRTR2* gene.

Methods. Familial Danish, Italian, and Swedish CH patients were ascertained as described previously.[18,33,34] Danish probands, recruited from two neurologic clinics by a single investigator

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Table 1 *Clinical characteristics of the cluster headache (CH) case-control cohort*

Cases	Danish	Italian	Swedish	UK
Sporadic cases, n	96	11	100	63
No. of chronic CH	6	4	4	N/K
No. of episodic CH	90	7	96	N/K
Male	65	9	76	43
Female	31	2	24	20
Mean age at onset, y (range)	31.8 (5–65)	23.9 (18–36)	29.7 (11–63)	N/K
Control subjects, n	72	0	106	89
Male	50	—	68	47
Female	22	—	38	42

Distinction between chronic and episodic CH and details of age at onset were not recorded for the UK cohort.

N/K = not known.

Table 2 *Clinical characteristics of cluster headache (CH) kindreds used in linkage analyses*

	Danish cohort	Italian cohort	Total
No. of families	21	12	33
Total no. of individuals genotyped	125	37	162
Mean no. of subjects per family (range)	6.0 (2–15)	3.1 (2–7)	4.9 (2–15)
Total no. of affected subjects genotyped	56	26	82
Mean no. of affecteds per family (range)	2.7 (2–6)	2.2 (2–4)	2.5 (2–6)
Total no. of chronic CH cases	6	0	6
Male	3	—	3
Female	3	—	3
Mean age at onset, y (range)	35 (18–56)	—	35 (18–56)
Total no. of episodic CH cases	50	26	76
Male	37	17	54
Female	13	9	22
Mean age at onset, y (range)	26.1 (7–52)	23.5 (10–50)	25.2 (7–52)

(M.B.R.) and through the Danish patient organization for CH, were asked to complete a mailed questionnaire detailing any known family history. Patients of Italian origin, ascertained through the Headache Centre of the Carlo Besta National Neurologic Institute, all underwent physical and neurologic examination including cerebral CT or MRI to exclude secondary headache. Swedish CH patients were collected at the Department of Neurology, Huddinge University Hospital, or via the CH network on the Swedish migraine patients' Web site (Svenska Migränförbundet; http://www.migran.org/), and all affected subjects were clinically examined by a neurologist specialized in headache (C.S.). At all three centers, probands and their relatives were interviewed either directly or by telephone to establish a family history of CH. The UK subjects were recruited via the Organisation for the Understanding of Cluster Headaches in the UK (OUCH [UK]; http://www.clusterheadaches.org.uk/). Criteria used for diagnosis of CH were consistent between each of the four countries and were as specified by the International Classification of Headache Disorders.[2] Peripheral blood samples were taken from all consenting familial CH patients (n = 167; 69 Danish, 43 Italian, 50 Swedish, and 5 UK) and their healthy relatives.

CH singletons used in the *HCRTR2* association analysis were ascertained in the same way as familial cases, but reported no family history of the condition. Control subjects, matched for ancestry, were drawn from the general population (Denmark) or consisted of blood donors, healthy volunteers, or spouses of probands (Sweden and UK). Table 1 summarizes the clinical characteristics of the case-control cohort.

All patient and control populations were of Caucasian origin. The study was approved by the relevant local ethics committees and all participants gave informed consent prior to the study.

DNA extraction and genotyping. Genomic DNA was extracted from 10 mL of peripheral venous blood using standard techniques. A genome-wide screen was performed for individuals from five multigenerational Danish families, using fluorescently labeled microsatellite markers with an average spacing of 9 cM (Marshfield Center for Medical Genetics, screening set v10). PCR amplifications were performed on MJ Research DNA Engines (10 μL reactions; 50 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs [Pharmacia], 0.2 μM each primer, 0.5 U *Taq* polymerase [ABgene] in a 10 × KCl PCR buffer [ABgene]). PCR products were pooled and electrophoresed through 6% polyacrylamide gels (Flowgen) on an ABI 377 DNA sequencer. Genotype data were generated using GeneScan v3.0 and Genotyper v2.1 software (Applied Biosystems). Data for 21 markers that failed to optimize were excluded from further analysis. Typing was successful for 95% of genotypes at the remaining 383 markers (full details available on request to author). Data for the X and Y chromosomes were used to confirm pedigree structures but were not included in linkage analyses, as the frequency of affected females and presence of male-to-male transmission were considered inconsistent with a sex-linked model of inheritance.

Following statistical analysis of the genome screen data, further markers (D2S2299, D2S142, D2S2380, D2S111, D2S130, D2S2390, D2S401, D2S2213, D8S1755, D8S552, D8S1827, D8S1731, D9S288, D9S1810, D9S281, and D9S286), selected from genetic maps at the Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/Map_Markers/maps/IndexMapFrames.html), were typed for those loci showing evidence of potential linkage. At this stage of analysis, the cohort was supplemented by inclusion of newly available material, namely a further 8 family members (3 affected) from families D42, D162, and D651, plus 111 additional individuals (60 affected) from 16 Danish and 12 Italian kindreds. Table 2 summarizes the characteristics of all CH kindreds used in linkage analyses.

For the SNP genotyping, DNA samples were arranged into 96-well microtiter plates, comprising 259 unrelated sporadic CH patients and 267 ethnically matched control subjects. Patients of Italian origin were omitted from the *HCRTR2* association analysis due to the small sample size and lack of a suitable control population (see table 1). Two polymorphisms of the *HCRTR2* gene (rs3122169 and rs2653349 from NCBI dbSNP; http://www.ncbi.nlm.nih.gov/SNP/) were amplified from plated DNA samples using sequence-analysis primers for exons 2 and 5, and resultant products were dot-blotted onto Hybond N (Amersham). SNPs were typed by ASO hybridization using a tetramethylammonium chloride (TMAC) hybridization protocol.[35] Alleles were assigned by direct examination of autoradiographs compared for each allele.

HCRTR2 sequencing. The complete protein-coding region and intron/exon boundaries, including splice donor/acceptor sites, 3′ UTR, and 200 bp of flanking 5′ UTR, of the *HCRTR2* gene were amplified by PCR in 40 μL reactions for an affected subject from each of eight independent families, namely D42, D162, ITA6, ITA48, SWE13, SWE14, UK1, and UK5, plus one normal control individual. PCR products were separated by electrophoresis through 2% LE agarose (FMC Bioproducts) to ensure the presence of sufficient quantities for sequence analysis, and were then purified using the QIAquick PCR purification kit (Qiagen). Purified PCR products were sequenced with the Applied Biosystems BigDye terminator kit, and were electrophoresed through 5% LongRanger (Cambrex) polyacrylamide gels on an ABI 377 automated sequencer (Applied Biosystems). Sequence traces were analyzed with Sequence Analysis v3.2 and SeqEd v1.0.3 computer software (Applied Biosystems).

Statistical analyses. Family-based data were first analyzed using the simulation program SLINK,[36,37] to establish the cumulative power to detect significant evidence for linkage contained in the five largest Danish families (D42, D150, D162, D651, and D799). These families were selected as they consisted of three or more affected individuals, distributed across at least one generation. Marker data were generated randomly for 1,000 replications, and simulated datasets analyzed under a disease model of autosomal dominant inheritance with a reduced penetrance of 30%. A

disease allele frequency of 0.001 and a phenocopy rate of 0.001 were assigned. Simulations were performed in duplicate, assuming locus homogeneity and heterogeneity, with percentages of linked families designated at 100% and 70%.

The computer program GAS (Genetic Analysis System v2.0; Alan Young, Oxford 1993–1995) was used to format genotype data for linkage analysis, to verify inheritance, and to calculate marker allele frequencies. Any identified inconsistencies were corrected by re-assessment of original GeneScan files and, if necessary, re-genotyping. If anomalies could not be resolved after a second round of genotyping, data for all family members were removed to avoid potential biasing.

Single- and multipoint linkage analyses were performed using Genehunter v2.1.[38] lod Scores were calculated using the disease model described above. Due to potential ambiguity for the mode of transmission in CH, Genehunter was also used to generate model-free nonparametric NPL scores for each analysis. For those loci reaching a lod or NPL score of 2.0 or above, corresponding to the criteria of potentially interesting,[39] four additional markers spanning each locus with an average spacing of 2 cM were next analyzed.

Association analysis of *HCRTR2* SNP data were assessed using a χ^2 test, to compare both allele and genotype frequencies between case and control populations. Hardy-Weinberg equilibrium was observed in CH cases and controls and significance was established at $p < 0.01$.

Results. *Linkage analysis.* We chose five extended kindreds to perform simulations of linkage using the SLINK program. Estimates of the maximum lod score, based on all markers and subjects being informative, achieved values of 3.35 and 3.17, under models of disease locus homo- and heterogeneity. Furthermore, the likelihood of reaching a combined lod score of >3 by chance alone was calculated as 1.6% of the replicates, which decreased to 0.2% if a model of more than one disease locus for CH was applied.

Using real genotype data, we performed single- and multipoint linkage analyses to generate lod and NPL scores for each autosome. The results from the complete genome screen gave no significant support for linkage to a single chromosomal region. However, lod and NPL scores either potentially interesting or suggestive of linkage (≥2.0) were obtained for two distinct loci on chromosome 2 (markers D2S1353; 164.51 cM, and D2S1363; 227.0 cM), and one locus on each of chromosome 8 (GATA151F02; 27.4 cM) and chromosome 9 (D9S2169; 14.23 cM). The figure illustrates the two-point lod and NPL scores combined with the multipoint linkage analyses.

For each of the loci demonstrating potential linkage, four additional markers were typed and genotype data analyzed for the whole chromosome to extract maximum information from the Genehunter analysis package. Of the four loci examined, inclusion of additional markers maintained support of potential linkage at the GATA151F02 locus on chromosome 8 alone. Multipoint linkage analysis generated NPL scores over 2.0 for three of the four flanking markers (NPL_{max} = 2.32 for distal marker D8S1827). Single point analysis failed to support linkage to newly typed markers flanking any of the initial loci investigated.

Genotype data for each of the four distinct potentially linked loci were next analyzed in 60 further subjects, ascertained primarily as nuclear kindreds with two or more affected individuals. Pedigrees were subdivided into Danish and Italian cohorts, to establish whether individual populations provided evidence of linkage to distinct loci, before repeating the analyses for the combined dataset. The Danish cohort (consisting of families D42 to D799 plus 16 new pedigrees) provided no evidence of linkage to any of the identified loci, generating a maximum combined multipoint NPL score of 1.82 at D8S1827, and a maximum single point NPL of 1.41 for GATA151F02. In contrast, analysis of a small Italian cohort generated compelling support for linkage to chromosome 9, with multipoint NPL scores of 2.19 and 2.41 (p = 0.0098) for markers D9S2169 and D9S281 observed. These scores were not maintained when Danish and Italian populations were combined.

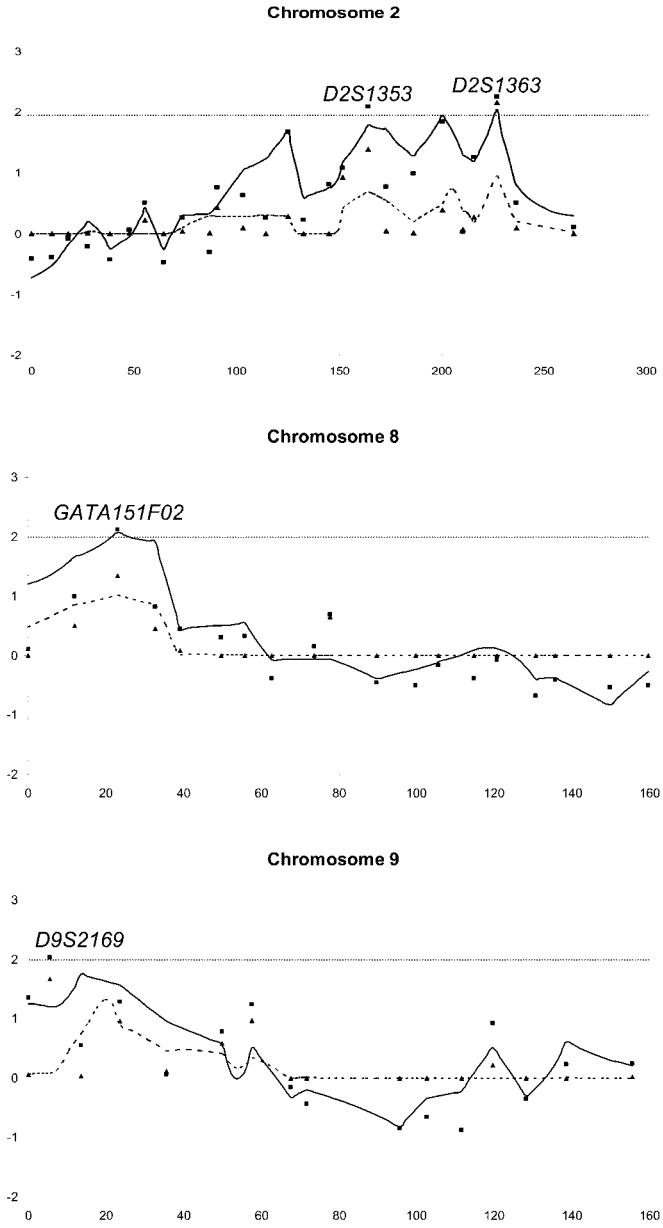

Figure. Graphs illustrating results of chromosome specific linkage analyses from genome-wide scan. Data identifying regions of potential linkage (designated as NPL/lod ≥ 2.0) are displayed, with distance along the chromosome in centiMorgans (x-axis) and lod/NPL score (y-axis). Solid lines represent multipoint NPL scores; dashed lines, multipoint lod scores. Filled squares illustrate two-point NPL scores; filled triangles, two-point lod scores.

Mutation analysis and association study of HCRTR2. To determine whether a pathogenic mutation of the *HCRTR2* gene might explain the familial occurrence of CH in our kindreds, we performed sequencing of

Table 3 *Genotype and allele frequencies of HCRTR2 single nucleotide polymorphisms in the complete cohort*

		GF (%)					AF (%)			
	n	AA	AC	CC	χ^2	*p* Value	A	C	χ^2	*p* Value
c.224-26A>C [rs3122169]										
CH patients	258	172 (66.7)	80 (31.0)	6 (2.3)	2.86*	0.239	424 (82)	92 (18)	0.273†	0.603
Controls	238	159 (66.8)	67 (28.2)	12 (5.0)			385 (81)	91 (19)		
c.922G>A [rs2653349]		GG	GA	AA			G	A		
CH patients	257	165 (64.2)	84 (32.7)	8 (3.1)	2.73*	0.255	414 (81)	100 (19)	1.80†	0.180
Controls	267	161 (60.3)	90 (33.7)	16 (6.0)			412 (77)	122 (23)		

* Two degrees of freedom.
† One degree of freedom.

GF = genotype frequencies; AF = allele frequencies.

genomic DNA for a single affected subject (total n = 8) from each of two independent families of Danish, Italian, Swedish, and British origin. No deleterious sequence variants were detected by comparison to wild-type control sequence.

Table 3 shows the genotype and allele frequencies observed for each of the *HCRTR2* single nucleotide polymorphisms examined. For the intronic SNP [rs3122169] we detected no significant difference in allele or genotype frequencies between the patient and control populations, using a χ^2 test of probability. Results for the 922G/A polymorphism in exon 5 [rs2653349], previously reported to be associated with CH, showed no difference in either genotype or allele frequencies in our diverse CH cohort, when compared with a matched control population. To establish whether the tested polymorphisms showed an association with CH in individual cohorts, the same data were stratified into distinct populations. Again, we detected no significant difference for either genotype or allele frequencies between CH patients and ethnically matched controls (table 4). Further stratification by sex, taking into account the sex bias observed in CH, also provided no support for association (data not shown).

Discussion. Anticipating the complexity of inherited predisposition to CH, we initiated a European collaboration to pool material from probands, sibships, and multiplex extended kindred ascertained across Northern Europe. To date, this cohort comprises subjects ascertained from 84 independent kindreds with CH (167 affected subjects) and 270 sporadic CH cases. In this study, we sought to identify gene locations implicated in disease predisposition by performing a medium (9 cM) density genome-wide scan. In addition, we sought replication of a specific candidate gene identified through an association study.

We assessed five multiplex kindreds, each of Danish origin, using a parametric model of a CH gene acting as an autosomal dominant trait with 30% penetrance. No significant lod scores were identified for any locus across the genome. We next performed a model-free (nonparametric) based analysis, and identified potentially interesting scores for four loci, namely chromosome 2, D2S1353; chromosome 2, D2S1363; chromosome 8, GATA151F02; and chromosome 9, D9S2169. To further interrogate these regions of putative interest, we next studied an extended collection of Danish and Italian kindreds, together with additional independent subjects. We typed further STS markers for each region and, despite adequate power to detect linkage under assumptions of homogeneity, no single locus reached significance. In conclusion, we were unable to specify

Table 4 *Stratification of SNP data into ascertainment populations*

	c.224-26A>C [rs3122169]						c.922G>A [rs2653349]					
		GF (%)			AF (%)			GF (%)			AF (%)	
	n	AA	AC	CC	A	C	n	GG	GA	AA	G	A
Danish cohort												
CH patients	96	61 (63.5)	33 (34.4)	2 (2.1)	155 (81)	37 (19)	96	56 (58.3)	38 (39.6)	2 (2.1)	150 (78)	42 (22)
Controls	69	40 (58.0)	26 (37.7)	3 (4.3)	106 (77)	32 (23)	72	37 (51.4)	31 (43.1)	4 (5.6)	105 (73)	39 (27)
Swedish cohort												
CH patients	99	72 (72.7)	24 (24.2)	3 (3.0)	168 (85)	30 (15)	98	68 (69.4)	26 (26.5)	4 (4.1)	162 (83)	34 (17)
Controls	84	60 (71.4)	19 (22.6)	5 (6.0)	139 (83)	29 (17)	106	67 (63.2)	32 (30.2)	7 (6.6)	166 (78)	46 (22)
UK cohort												
CH patients	63	39 (61.9)	23 (36.5)	1 (1.6)	101 (80)	25 (20)	63	41 (65.1)	20 (31.7)	2 (3.2)	102 (81)	24 (19)
Controls	85	59 (69.4)	22 (25.9)	4 (4.7)	140 (82)	30 (18)	89	57 (64.0)	27 (30.3)	5 (5.6)	141 (79)	37 (21)

GF = genotype frequencies; AF = allele frequencies.

a solitary disease locus for the CH phenotype using the available dataset.

CH rarely occurs in families as a monogenic trait and our data might suggest locus heterogeneity underlying inherited predisposition to familial CH. This observation would parallel similar findings in other forms of genetically determined headache. For example, molecular genetic studies have identified mutations in at least two causative genes, *CACNA1A* and *ATP1A2*, in familial hemiplegic migraine, a rare autosomal dominant form of migraine associated with aura.[24,40] However, recent genome-wide screens in common migraine suggest other, as yet uncharacterized, genes may contribute to this group of disorders, with identification of susceptibility loci on chromosomes 4q, 6p12.2-p21.1, 11q24, 14q21.2-q22.3, 15q11-q13, and Xq24-q28,[41,42] together with evidence of suggestive linkage to chromosomes 3q and 18p11.[43]

The discovery that inherited forms of headache can develop through disruption of specific ion channels provides important insight as to mechanistic defects of potential relevance to CH and thereby suggest numerous candidate genes and loci for examination. However, a number of clinical features and characteristics typical of CH suggest the likely involvement of alternative pathways underlying disease predisposition. For example, the periodicity of attacks implies a potential defect within the suprachiasmatic nucleus of the hypothalamus, a key neurogenic regulator of the human biologic clock and which has been shown to become activated during a cluster attack.[10] Furthermore, the severity of pain associated with CH compared to other types of headache might suggest a unique pathophysiology for this disorder. A recent report of association between a polymorphism in the hypocretin receptor gene, *HCRTR2*, in a cohort of sporadic CH patients ascertained within Italy, might provide additional evidence for the role of genetic factors in disease predisposition.[32] However, there is now compelling evidence that initial candidate gene association studies carry a high likelihood of false-positive results, an observation that stresses the need for balanced publication of replication studies that might (rarely) confirm or (commonly) reject novel association results.[44] Here we examined two polymorphisms which include the associated coding sequence variant (c.922G>A) in a larger sample size, with greater than 95% power to detect association under identical conditions of replication. We detected no significant support for allelic association at the *HCRTR2* locus. Stratification of association data into discrete populations failed to yield evidence for association of either SNP with CH. Furthermore, we found no evidence of linkage to chromosome 6p12.1 within our family based genome-wide scan. Finally, we performed sequence analysis of genomic DNA for the entire coding region of *HCRTR2* in eight probands from CH families and detected no pathogenic sequence variants. Hence, and in contrast to the original report, we find no evidence for even marginal effects of variants within the *HCRTR2* gene in the European cohorts investigated here. However, our study cannot exclude the possibility that other variants may be either disease causing or in linkage disequilibrium with disease causing variants that are specific to one or more populations. In this regard, it should be noted that the association analysis did not include any subjects ascertained in Italy as we do not have an adequate population-based control cohort for that region. The potential for hidden population stratification as an explanation for the conflicting results from the studies looking at association between *HCRTR2* and CH is further underlined when we note the marked variation in allele frequencies of this gene in different control cohorts (see table 4).[32] At this stage of investigation into the genetic basis of CH, our findings argue strongly for careful attention to methodologic detail. For example, further linkage studies will require large sample sizes or extended pedigrees with multiple affected subjects, ideally ascertained prospectively to deal with inherent concerns of historical records of the CH phenotype in previous generations. For association studies, this work adds further to the call for collaboration between centers working in this field as sample size and independent replication within a study should be the gold standard of future reports.[45]

This report suggests that genetic predisposition to CH is liable to be complex and likely compounded by locus heterogeneity and variation in genes conferring only a small effect size. Despite recent progress in therapeutic strategies for the management of CH, a detailed understanding of the etiology and pathogenesis remains the most plausible means of comprehensive and targeted approaches to the treatment of this debilitating disorder.

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